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Award Number: W81XWH-10-1-0636

TITLE: Nanog, Cancer Stem Cells, and Resistance to Chemotherapy

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CONTRACTING ORGANIZATION: Southern Illinois University
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REPORT DATE: September 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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# REPORT DOCUMENTATION PAGE

*Form Approved*
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| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| 01-09-2011 | Annual Summary | 1 SEP 2010 - 31 AUG 2011 |

**4. TITLE AND SUBTITLE**
Nanog, Cancer Stem Cells, and Resistance to Chemotherapy

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-10-1-0636

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Ms. Hongmei Jiang

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Southern Illinois University
Springfield, IL 62702

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
No abstract provided.

**15. SUBJECT TERMS**
No subject terms provided.

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 14 | **19b. TELEPHONE NUMBER** *(include area code)* |

**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

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## Introduction

It is now increasingly accepted that cancer stem cells (CSCs, or tumor initiating cells) are responsible for tumor initiation. If cancer treatment kills most of cancer cells in the stage of transit amplifying and differentiation without killing the stem cells, the surviving cancer stem cells will eventually lead to recurrence of tumors. To eradicate cancer, we must learn more about the biology of cancer stem cells, their responses to treatments, and their role in tumor recurrence after treatment. In the preliminary studies, I found that Nanog, a transcription factor essential for self-renewal of embryonic stem cells, was expressed in prostate cancer cells, and further its expression was associated with tumor cells positive for stem/progenitor markers. Knockdown of Nanog reduced the ability of cancer cell to form tumors in an animal model. I further found that tumor cells with endogenous Nanog expression were particular resistant to chemotherapy. The data suggest that Nanog is associated with prostate cancer stem cells and Nanog may cause resistance toward chemotherapy.

Based on the preliminary data, it was hypothesized that Nanog promotes resistance of prostate carcinoma cells toward chemotherapy and that Nanog, or its downstream effectors, should be targeted for eradication of tumorigenic prostate carcinoma cells. To test my hypothesis, the following specific aims are proposed:

1) To define the role of Nanog in resistance of prostate carcinoma cells toward chemotherapy.

2) To determine whether Nanog can be targeted to eliminate the chemoresistance of prostate cancer cells.

3) To elucidate the mechanism of Nanog-mediated chemoresistance.

## BODY OF REPORT

**Scientific portion:**

**Task 1. To define the role of Nanog in resistance of prostate carcinoma cells toward chemotherapy. (Months 1 – 12).**

Increased expression of Nanog in the surviving fractions of prostate cancer cells after chemotherapy: As a transcription factor essential for self-renewal of embryonic stem cells, Nanog has been found to be expressed in prostate cancer cells and further it regulates tumor development (1) and essential for prostate cancer cells to initiate tumor formation (Appendix 1). To determine whether Nanog plays a role in prostate cancer drug resistance, we first examined Nanog protein level in the surviving fractions of tumor cells after treatment with different chemotherapeutics. There was a higher level in Nanog protein in the surviving fractions of LNCaP cells treated with 10 nmol/L of Taxol, or 0.3 nmol/L vinblastine for 40 hours, when compared to those not treated (NA) or treated with DMSO (**Figure 1**, top panel). N-Tera cells were used as a positive control for western blot. In the surviving fractions of DU145 cells treated with vinblastine or doxorubicin, there were increased levels of Nanog protein (**Figure 1**, bottom panel). The results suggest that there were increased levels of Nanog protein in the surviving fractions of prostate cancer cells after chemotherapy.

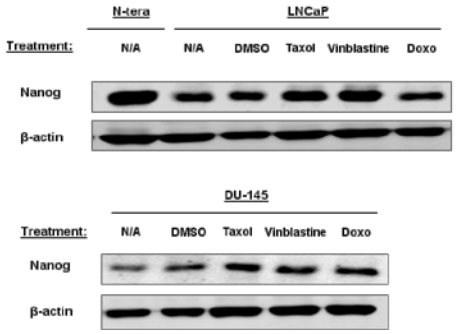


**Figure 1.** Increased Nanog protein level and promoter activities in the surviving fractions of prostate cells after chemotherapy shown by Western blot analysis. Note the increased Nanog levels in surviving fractions from Taxol or vinblastine treatment.

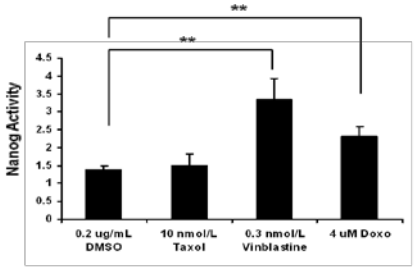


**Figure 2.** *NANOG1* promoter activities in DU145 cells after chemotherapy. The cells were transfected with *NANOG1* promoter luciferase reporter, and then treated with different therapeutic drugs. The surviving cells were harvested and assayed for luciferase activities. Note the increased NANOG1 promoter activities in cells treated with vinblastine or doxorubicin (N=3; ** $P < 0.01$).

Nanog has more than eleven pseudogenes (2). It has been suggested that the pseudogene *NANOGP8* is expressed at mRNA level in cancer cells (1), but one of our recent studies suggest that it is the *NANOG1* gene loci that is responsible for Nanog expression in tumorigenic prostate

cancer cells (3). To determine whether *NANOG1* promoter activities were increased after chemotherapy, LNCaP or DU145 cells were transfected with a *NANOG1* promoter luciferase reporter construct and then the cells were treated with different chemotherapeutics for 24 h and then the luciferase activities in the surviving fractions were assayed. As shown in **Figure 2**, the *NANOG1* promoter activities were increased in the DU145 cells after treatment with vinblastine or doxorubicin. The results suggest that the *NANOG1* promoter activities were either enriched in the surviving fractions of tumor cells after chemotherapy, or activated by treatment of chemotherapeutics.

Prospective enrichment of tumor cells with *NANOG1* promoter activities: To determine a possible functional role of Nanog in chemoresistance, we marked and selected tumor cells with active *NANOG1* promoter activities using a reporter construct in which expression of GFP and zeocin resistance is under the control of 2.5 kb *NANOG1* promoter (pGZ-Nanog). We enriched the cells with active *NANOG1* promoter activities using zeocin selection. After selection, most of zeocin-resistant cells transduced with pGZ-NANOG were GFP positive (**Figure 3**). When compared to the vector control, tumor cells enriched with active *NANOG1* promoter activities tended to form sphere-like structures (**Figure 3**). Western blot analysis revealed that the selection of cells with *NANOG1* promoter activities led to an enrichment of cells with higher endogenous NANOG expression at protein level (**Figure 4**), further suggesting a role of *NANOG1* in the endogenous expression of NANOG protein.

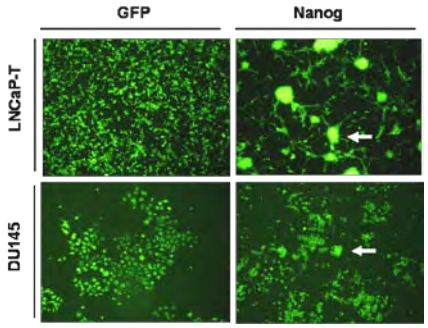


**Figure 3.** DU145 or LNCaP-T cells stably enriched with *NANOG1* promoter activities. Note the sphere-like structures in cells enriched with *NANOG1* (right panel), as compared to the vector control.

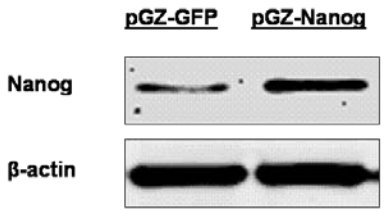


**Figure 4.** Western blot analysis of Nanog levels in Nanog-enriched cells vs. vector controls. Densitometry analysis revealed an approximate one fold increase in Nanog.

Tumor cells selected for active *NANOG1* promoter activities had increased expression of stem/progenitor markers: Normal prostate stem cells or prostate tumor stem cells have been identified as cells with high surface expression of integrin $\alpha_2\beta_1$, CD44, and CD133 (4) (5, 6). To determine whether Nanog expression marks a subpopulation of tumor cells with stem cell property, we analyzed the expression of stem cell markers $CD133^+/CD44^+$, in cells enriched for NANOG endogenous expression. An increase in CD133 protein levels in DU145 and LNCaP-T cells enriched with NANOG expression was found by Western blot (**Figure 5**). In addition,

increased CD44 surface expression was found in LNCAP-T cells enriched with NANOG expression as well as in DU145 cells enriched with NANOG expression (**Figure 6**).

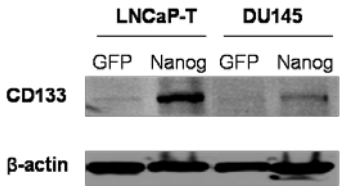


**Figure 5.** Increased levels of CD133, a marker of stem cells, in tumor cells with active NANOG1 promoter activities.

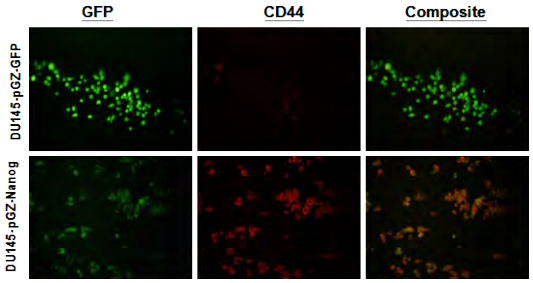


**Figure 6.** Increased levels of CD133 (Red color), a marker of stem cells, in tumor cells with active NANOG1 promoter activities.

Increased resistance toward chemotherapy by tumor cells with active *NANOG1* promoter activities: The above studies suggest that NANOG marks prostate cancer cells with stem cell markers. To determine whether tumor cells with endogenous Nanog expression are inherently resistant to chemotherapy, we evaluated the responses of Nanog-enriched cells toward several chemotherapeutics, in comparison with the vector control cells. As shown in **Figure 7**, the enriched Nanog-expressing LNCaP cells also presented an increased resistance toward taxol.

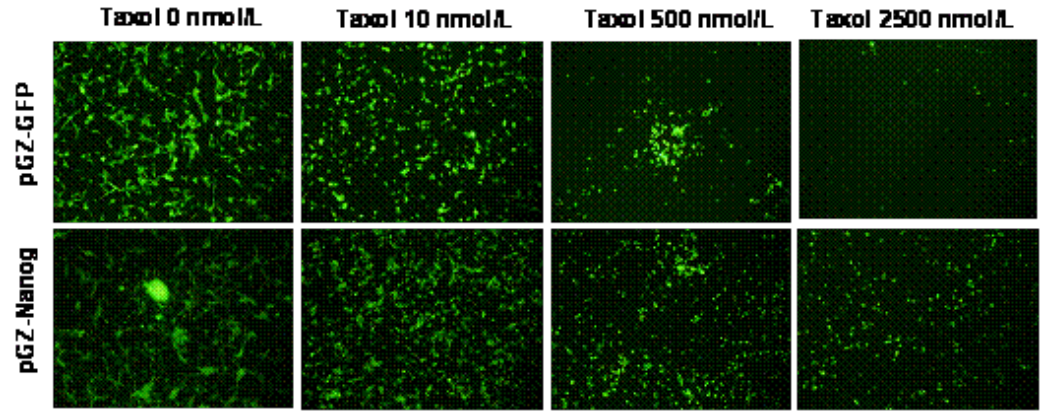


**Figure 7.** The cell morphology 24 h after taxol treatment: Most LNCaP-pGZ-GFP cells were sensitive to the taxol treatment at the concentration of 500 and 2500 nmol/L. The green cells indicate the remaining viable cells in culture after treatment.

As shown in **figure 8**, DU145 cells enriched with Nanog expression presented increased resistance to doxorubicin. The results suggest that enrichment of tumor cells with Nanog expression also enrich drug resistant cells. Here we propose to extend our preliminary studies on NANOG-mediated resistance toward chemotherapy.

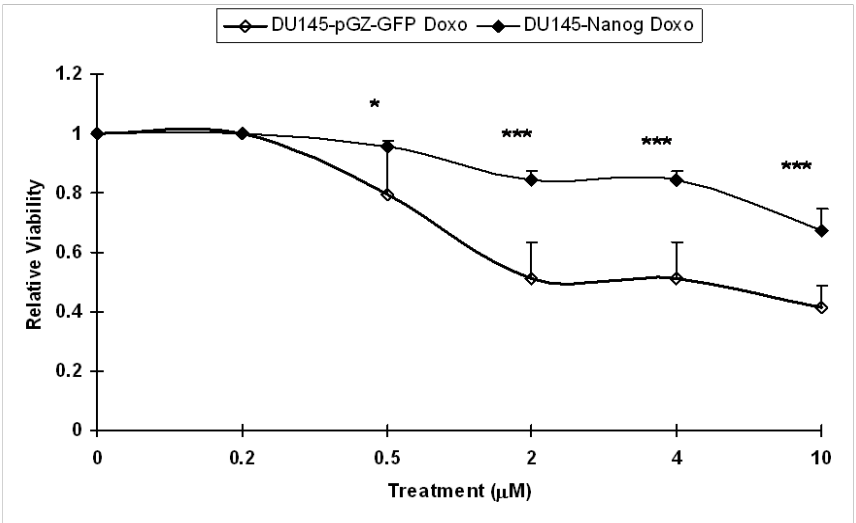


**Figure 8**. The Nanog-enriched cells exhibited enhanced resistance to doxorubicin in comparison with pGZ-GFP control cells. DU145-pGZ-GFP and –Nanog cells were treated with DMSO or doxorubicin for 72 hours, and the cell viability was measured by MTS assay. (N = 6; *** P < 0.001; * P < 0.05).

Forced expression of Nanog in prostate cancer cells: To determine whether Nanog expression is sufficient to render prostate cancer cells resistant to chemotherapy, I overexpressed Nanog in DU145 cells using a lentiviral vector. DU145 cells were infected with piPSC-hNanog or its vector control and the expression of Nanog was determined by Western blot analysis. As shown in **Figure 9**, DU145 cells infected with piPSC-hNanog had significantly increased Nanog protein. We are currently generating and characterizing stable sublines with Nanog stably overexpressed. Once we obtain them, we will evaluate whether forced expression of Nanog cause resistance toward chemotherapeutics.

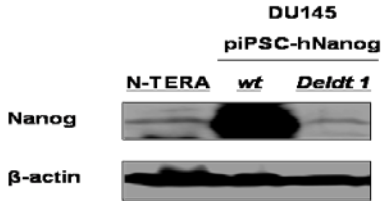


**Figure 9**. Validation of antibody used for Western blot analysis of Nanog protein. N-Tera cells were used as positive control. The antibody is validated by the observed large increase in Nanog protein levels after infection with the Nanog expressing virus (wt, middle panel), but not in cells infected with the vector control (Deldt1).

**Task 2. To determine whether Nanog can be targeted to eliminate the chemoresistance of prostate cancer cells (Months 9 - 24).**

To determine whether Nanog can be a target to reduce resistance toward chemotherapy, we attempted to knock down the expression of Nanog using small hairpin RNAs and examined the resultant effects on tumor cell responses toward chemotherapy. It was found that infection of DU145 cells with a shRNA construct targeting Nanog (7) led to a decrease in Nanog protein level (**Figure 10**). The knockdown of Nanog increased the sensitivity of DU145 cells toward Taxol (**Figure 11**), vinblastine (**Figure 12**) and doxorubicin (not shown).

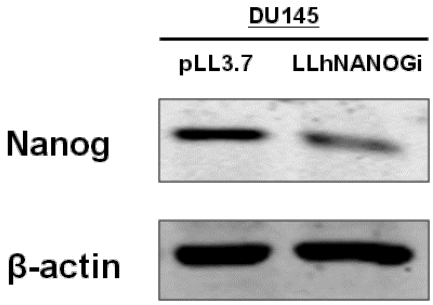


**Figure 10.** Western blot confirmation of the knockdown of Nanog in DU145 by shRNA.

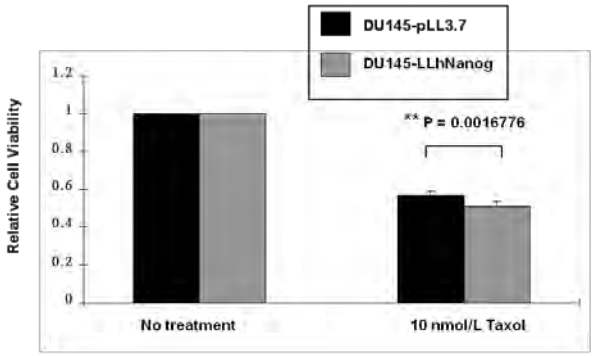


**Figure 11.** Increased sensitivity toward taxol by Nanog knockdown. The viability of surviving fractions was measured by MTS assay.

We further determined whether tumor cells with Nanog knocked down can be selectively eliminated by chemotherapy due to their increased chemosensitivity. Since the shRNA construct or its vector pLL3.7 utilized in Nanog knockdown also encodes GFP, we monitored the presence of cells with GFP positivity in the surviving fractions after chemotherapy. Parental DU145 cells were mixed with DU145 cells with Nanog knocked down or vector control cells, treated with different chemotherapeutics, and GFP positive cells in the surviving fractions were quantified by flow cytometry. If Nanog knockdown had no effects on the tumor cell sensitivity toward chemotherapeutics, we expect that GFP positive cells were still 50% in the surviving fractions. As shown in **Figure 13**, for DU145 cells infected with pLL3.7 (vector controls), there was a slight increase (more than expected 50%) in GFP positive cells in the surviving fractions. In contrast, in DU145 cells infected with Nanog knocking down LL-hNANOGi, there was a significant decrease in GFP positive cells in the surviving fractions after treatment with Taxol, vinblastine, or doxorubicin. The data suggest that tumor cells with Nanog knocked down were selectively eliminated. The increased sensitivity toward chemotherapy in tumor cells with Nanog knock down suggest an essential role for Nanog for tumor cells to resist chemotherapy.

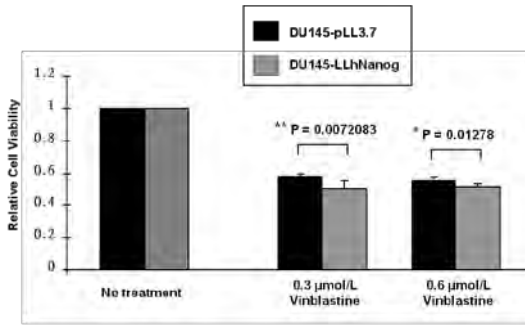


**Figure 12.** Nanog knockdown increased DU145 sensitivity toward vinblastine. Cell viability was measured by MTS assay.

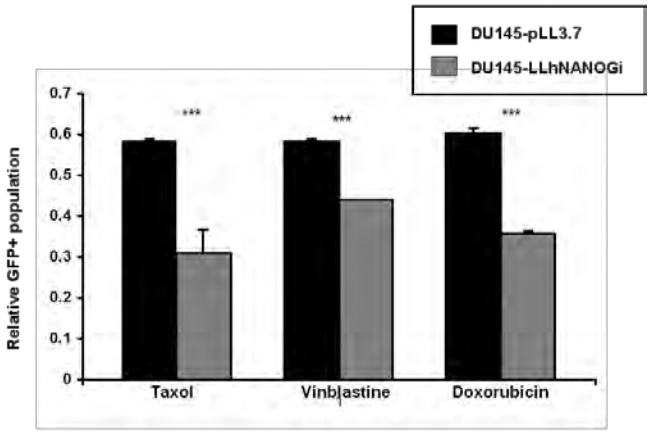


**Figure 13.** Selective elimination of Nanog knockdown cells by chemotherapy. Parental DU145 cells were mixed with GFP positive pLL3.7 DU145 cells or GFP positive LLhNANOGi DU145 cells in 1:1 ratio, and the mixed cells were treated with 0.2 µg/mL DMSO (as control), 10 nmol/L Taxol, 0.3 nmol/L Vinblastine as well as 6 µM Doxorubicin for 36 hours. The surviving cells were harvested for flow cytometry analysis for GFP positive cells (N = 3; *** P < 0.001).

**Aim 3. To elucidate the mechanism of Nanog-mediated chemoresistance. (Months 18 - 36).**

To be initiated.

**Training portions**

In the last year, the original PI, Man-Tzu Wang, obtained her Ph.D. degree. Now she is a postdoctoral fellow in Dr. Frank McCormick lab at UCSF Cancer Center.

The new PI, Ms. Hongmei Jiang, has had the following trainings:

A. Research-related training by learning all laboratory techniques required to complete the proposed studies, including, but not limited to: (Month 5 – 36)

Extraction of large plasmids more than 10 kb, cell culture, packaging of viral vectors, generation of stable cell lines with Nanog expressed or knocked down, FACS, evaluation of tumor cell responses to chemotherapy using MTS, trypan blue exclusion, and colony formation assays, Western blot, RNA isolation and cDNA synthesis, and statistical analysis.

B. Non-research tasks important for PI's career development:

B1. Oral presentations:

1) Presentations of research progresses in the lab meeting weekly. Ms Jiang has presented research findings in the lab meeting on weekly basis.
2) Presentations at student seminars. Ms. Jiang has given a seminar on her research findings in the spring. The audience is made up with students in the MBMB programs, faculty, and other interested researchers.
3) Presentation at scientific meetings. Ms Jiang and Wang presented the research findings in 2011 IMPACT meeting sponsored by DoD PCRP.

B2. Scientific writing skills:

1) Writing and submission of the annual progress report to DoD. (Every year)
2) Writing of research protocols or experimental approaches. (Every year)

## KEY RESEARCH ACCOMPLISHMENT and REPORTABLE OUTCOMES

### Presentations:

Man-Tzu Wang and Daotai Nie. Nanog, cancer stem cells, and resistance to chemotherapy. 2011 DoD PCRP Impact Meeting, Orlando, March 2011.

### Abstracts published:

Man-Tzu Wang and Daotai Nie. Nanog, cancer stem cells, and resistance to chemotherapy. Proceedings of the 2011 DoD PCRP Impact Meeting.

### Articles published:

Not yet. A manuscript is in the process of preparation.

**Conclusions and significance (So what?):**

Identification of key factors for tumor resistance to chemotherapy can lead to better strategy in cancer treatment. Our studies suggest that Nanog, a transcription factor essential for the self-renewal of embryonic stem cells, is expressed in tumorigenic cancer cells and further Nanog expression was enriched in the surviving fractions of tumor cells after chemotherapy. Knockdown of Nanog sensitized prostate cancer cells toward chemotherapy. Our studies suggest that Nanog can be targeted to improve the efficacy of chemotherapy of prostate cancer.

## APPENDICES

N/A

## SUPPORTING DATA

Embedded in the reporting body

## REFERENCES

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